Synergistic effect of Toll-like receptor 4 and CD14 polymorphisms on the total atherosclerosis burden in patients with peripheral arterial disease

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Background: **Genes involved in the regulation of immune responses, such as Toll-like receptor 4 (TLR4) and CD14, show genetic variations with potential functional implications. Because atherosclerosis is an inflammatory process apparently modulated by chronic infections, we studied the effect of single nucleotide polymorphisms (SNPs) in TLR4 and CD14 on the extent of clinically relevant atherosclerosis in patients with peripheral arterial disease (PAD).**

Methods: **Using an in-house–developed polymerase chain reaction–based restriction length polymorphism assay, we determined the genotype, allele frequency, and carrier traits of the *TLR4 +896 A>G* and the *CD14 −260 C>T* SNPs in 607 white Dutch patients with PAD. The extent of clinically relevant atherosclerosis was determined on the basis of the number of vascular territories involved, ie, coronary, cerebral, aortic, and peripheral.**

Results: **A total of 55% of the patients had PAD only. Approximately one third of the patients had two and 11% had three vascular territories affected by clinically relevant atherosclerosis. The *TLR4 +866 G* allele frequency was 11%, and the *CD14 −260 T* allele frequency was approximately 74%. Among PAD patients, *TLR4 +896 G* allele carriership was univariantly associated with extensive (more than two vascular territories affected) atherosclerotic disease (odds ratio, 2.22; $P = .020$; χ^2 test), whereas *CD14 −260 C>T* carriership/homozygosity was not. Trend analysis showed that the *TLR4 +866 G* allele frequency increased with the number of vascular territories affected by clinically relevant atherosclerosis (*P* trend, .0074). In a multivariate logistic regression analysis including cardiovascular risk factors and *TLR4* and *CD14* SNPs, only the interaction variable "*TLR4 +896 G* allele carriership/*CD14 −260 TT* genotype" survived as an independent predictor of extensive atherosclerotic disease ($P = .031$; odds ratio, 4.2; 95% confidence interval, 1.1-15.4).**

Conclusions: **The carrier trait *TLR4 G* allele/*CD14 TT* genotype, rather than each SNP individually, is associated with the extent of clinically relevant atherosclerotic disease. Considering the importance of immune responses in atherogenesis and the genetic variation of immune regulatory genes, our data provide an explanation for interindividual differences in susceptibility to atherosclerosis and demonstrate the need to take a wider approach in analyzing relevant carrier traits instead of individual polymorphisms in relation to atherosclerosis. (J Vasc Surg 2006;44:326-32.)**

Inflammatory processes have been implicated in the pathogenesis of atherosclerosis.[1] Responses of the innate immune system to endothelial injury are involved in the initiation of atherosclerosis, and inappropriate activation of the innate and acquired immune system plays a pivotal role in the propagation of the disease.[2] Among the triggers of such atherogenic immune responses are endogenous antigens, such as oxidized low-density lipoprotein, and exogenous pathogen-associated molecular patterns, such as bacterial lipopolysaccharide (LPS) and exogenous and endogenous heat shock proteins. Indeed, chronic infections, especially *Chlamydia pneumoniae* infections, have recently been implicated in the pathogenesis of atherosclerosis.[3]

The innate immune system plays a key role in the defense against pathogens and, when gone awry, may contribute to the development of chronic inflammatory conditions. Pattern-recognition receptors such as Toll-like receptors (TLRs) are involved in the elimination of pathogens through recognition of pathogen-associated molecular patterns, which set off a cascade of proinflammatory reactions; if not balanced, these may exacerbate chronic inflammatory processes. TLR4 is the first TLR described in mammals[4] and functions as a receptor not only for LPS, but also for agonists such as human and chlamydial heat shock proteins.[5] LPS binding is complex and requires several accessory molecules, among which are CD14, LPS-binding protein, and MD-2.[6]

Candidate gene approaches investigate genetic variation, including the effect of (functional) single nucleotide polymorphisms (SNPs) in genes involved in the immune response on disease susceptibility, severity, or both. In

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polygenic and multifactorial diseases such as atherosclerosis, these approaches might identify risk factors in the context of etiologic and genetic heterogeneity. Potential candidate genes for investigating the susceptibility to and severity of atherosclerosis include pattern-recognition receptors such as TLR4 and CD14. Activation of these receptors results in the activation of nuclear factor-κB, followed by transcription of various proinflammatory cytokine genes such as tumour necrosis factor α, interleukin 1α, and interleukin 1β. Thus, genetic variations in pattern-recognition receptors provide a plausible explanation for altered responsiveness of the innate immune system and may be associated with altered susceptibility to infectious and inflammatory processes and with the severity or outcome of disease. The recently described human *TLR4* polymorphism, ie, a missense SNP substituting an aspartic acid residue with glycine at amino acid 299 (Asp299Gly; nucleotide position *TLR4 +896 A>G*), has been associated with hyporesponsiveness to LPS and reduced expression of TLR4.[7] In the general population, this polymorphism has been associated with decreased interleukin 6, fibrinogen, soluble vascular adhesion molecule 1, procalcitonin, and neopterin plasma levels, and it seemingly confers protection against the development of carotid and femoral atherosclerosis[8,9] and acute coronary events.[10] Among pravastatin users, it has been associated with a significantly lower cardiovascular risk.[11] A functional polymorphism in the promotor region of *CD14* at position −260, the *CD14 −260 C>T* polymorphism, which enhances the transcriptional activity of the *CD14* gene, has been associated with increased carotid artery intima media thickness[12] and an enhanced risk of stroke[13] and acute myocardial infarction.[14,15] Nevertheless, several authors have demonstrated a lack of association between this polymorphism and coronary artery disease (CAD) or cerebrovascular disease (CVD).[16-18]

Although candidate gene approaches may identify signal transduction pathways of importance for a specific disease, they usually find only relatively small contributions for an individual gene to the overall susceptibility to disease. Therefore, so-called carrier trait analyses are increasingly used. These strategies analyze SNPs in different genes together and investigate whether potential synergic effects can be observed. A good example of the effect of such analyses is shown in a recent study by El-Omar et al,[19] who evaluated the role of proinflammatory cytokine gene polymorphisms in gastric and esophageal cancers. They showed that combined carriage of multiple proinflammatory polymorphisms of interleukin 1B, interleukin 1 receptor antagonist, tumor necrosis factor α, and interleukin 10 conferred greater risk, with odds ratios (and 95% confidence intervals) of 2.8 (1.6-5.1) for one, 5.4 (2.7-10.6) for two, and 27.3 (7.4-99.8) for three or four high-risk genotypes.

In this study, we assessed the association between the *TLR4 +896 A>G* and *CD14 −260 C>T* polymorphisms individually and the extent of atherosclerosis in patients with peripheral arterial disease (PAD). Furthermore, using a multivariate logistic regression model, we analyzed the effect of the combination of both polymorphisms on the extent of atherosclerotic disease in these patients.

MATERIALS AND METHODS

Study population. White Dutch patients with symptomatic PAD were recruited at the surgical clinics of a university hospital and two affiliated teaching hospitals. Ankle-brachial pressure index (ABPI) measurement was used to objectify the presence of atherosclerotic disease of lower limb arteries. An ABPI less than 0.9 was regarded as pathognomonic. To identify additional cardiovascular and cerebrovascular comorbidities, medical charts were reviewed, and attending physicians were consulted. Also, the presence/absence of abdominal aortic aneurysm (AAA) was ascertained in all patients by means of duplex ultrasonography, computed tomographic angiography, or both. AAA was defined as an aorta with an anteroposterior diameter greater than 30 mm.

The study was approved by the local medical ethical committees of all participating centers and conformed with the principles outlined in the Declaration of Helsinki.[20] All patients gave written informed consent. Patients with acute infections, recent antibiotic use (<3 months), recent vascular surgery (<3 months), concomitant inflammatory disorders, and malignancies were excluded, because polymorphisms in innate immunity genes have been associated with these conditions.

Extent of atherosclerotic disease. We wanted to explore the relationship between the TLR4 and CD14 polymorphisms and clinically relevant atherosclerosis. The chosen outcome measure (extent of clinically relevant atherosclerosis) should take into account the systemic nature of atherosclerosis and incorporate only clinically relevant manifestations of atherosclerotic disease. Therefore, the extent of clinically relevant atherosclerotic disease was determined on the basis of symptomatic PAD, symptomatic CAD, symptomatic CVD, and clinically relevant AAA. Symptomatic PAD was defined as (a history of) intermittent claudication or critical limb ischemia accompanied by a decreased ABPI (<0.9). Symptomatic CAD was defined as a history of (unstable) angina pectoris; acute, healing, or healed myocardial infarction diagnosed according to the recommendations of The Joint European Society of Cardiology/American College of Cardiology Committee[21]; or coronary intervention (percutaneous transluminal coronary angioplasty or coronary artery bypass grafting). The diagnosis of myocardial infarction was based on the presence of chest symptoms, increased cardiac enzymes, and characteristic electrocardiogram changes in at least two contiguous leads and was made by the attending cardiologist/physician. Symptomatic CVD was defined as (a history of) amaurosis fugax, transient ischemic attack, or stroke and/or carotid endarterectomy. The diagnosis was made by the attending neurologist and was based on clinical symptoms and characteristic changes on serial cerebral computed tomography, magnetic resonance imaging, or both. Clinically relevant AAA was defined as an infrarenal aortic dilatation of at least 3 cm that merited further follow-up or a history of aortic reconstruction for AAA.

To determine the extent of clinically relevant atherosclerotic disease, an extent-of-atherosclerosis score was developed ascribing a point for every vascular territory (coronary, cerebral, peripheral, and aorta) affected by symptomatic atherosclerotic disease as defined previously. The sum, ranging from 1 (only PAD) to 4 (all vascular territories affected), was considered as an indication of the extent of atherosclerotic disease.

DNA extraction. Genomic DNA was extracted from peripheral blood mononuclear cells by using the isopropanol isolation method. Briefly, 600 μL of Nuclisens Lysis buffer containing 5 mmol/L guanidine thiocyanate, Triton X-100, Tris-HCl (Organon Teknika, Boxtel, The Netherlands), and 1 μL of glycogen was added to 100 μL of peripheral blood mononuclear cells in phosphate-buffered saline. The DNA pellets were dissolved in T10 (10 mmol/L Tris-HCl; pH 8.0) and stored at −20° C until further analysis.

Genotyping of *TLR4* and *CD14*. An in-house–developed polymerase chain reaction (PCR)-based restriction fragment length polymorphism assay was used to detect the A>G missense mutation at nucleotide 896 base pairs (bp; amino acid Asp299Gly) in the human *TLR4* gene (National Center for Biotechnology Information SNP cluster ID rs4986790).[22] Digestion with *Nco*I (Invitrogen Live Technologies BV, Breda, The Netherlands) and separation on a 4.5% agarose gel containing 0.1% ethidium bromide (BIOzymTC, Landgraaf, The Netherlands) of the 102-bp PCR product (primers 5′-AGC ATA CTT AGA CTA CTA CCT CCA TG-3′ and 5′-TTT ACC CTT TCA ATA GTC ACA CTC A-3′) yielded fragments of 102 bp (A allele) and/or 80 and 22 bp (G allele).

The C>T substitution in the proximal CD14 promotor region at position −260 (National Center for Biotechnology Information SNP cluster ID rs2569190) was analyzed with an in-house–developed PCR assay with the primers 5′-TCA CCT CCC CAC CTC TCT T-3′ (sense) and 5′-CCT GCA GAA TCC TTC CTG TT-3′ (antisense; Invitrogen Live Technologies BV). The 107-bp amplification products were digested with *Hae*III (New England Biolabs, Hitchin, UK) and yielded two fragments of 83 and 24 bp (C allele) and/or an intact fragment (T allele) of 107 bp. Both assays are 100% reliable and reproducible.

Statistics. The χ^2 test was used for comparison of the *TLR4 +896 A>G* and *CD14 −260 C>T* genotype frequencies, carrier trait analyses (combined effect of the *TLR4* and *CD14* polymorphisms), and prevalence of risk factors between patient groups. A trend analysis was used to investigate whether specific CD14 or TLR4 allele frequencies increased with the number of vascular territories affected by atherosclerosis. Multivariate logistic regression models were computed by using extensive atherosclerotic disease (ie, more than two vascular territories affected by atherosclerosis) as a dependent variable and entering, in a stepwise forward conditional fashion, cardiovascular risk factors, *TLR4* and *CD14* SNPs, and the *TLR4/CD14* SNP interaction variable as independent variables. SPSS 10.0 for Windows (SPSS Inc, Chicago, Ill) was used for statistical analysis.

Table I. Patient characteristics (n = 607)

Variable	*Data*
Mean (SD) age (y)	65 (10)
Female/male	184 (30%)/423 (70%)
Smoking*	476 (78%)
Dyslipidemia†	503 (83%)
Hypertension‡	363 (60%)
Diabetes§	138 (23%)
Peripheral arterial disease	607 (100%)
Coronary artery disease	195 (32%)
Cerebrovascular disease	88 (14%)
Abdominal aortic aneurysm	67 (11%)
One vascular territory affected (PAD only)	334 (55%)
Two vascular territories affected (PAD + one other territory)	204 (34%)
Three vascular territories affected (PAD + two other territories)	67 (11%)
Four vascular territories affected (PAD + three other territories)	2 (0%)

PAD, Peripheral arterial disease.
*Currently smoking or stopped for <10 years.
†Fasting cholesterol level >6.5 mmol/L and/or triglyceride level >1.95 mmol/L and/or the use of antidyslipidemic medication.
‡Systolic blood pressure >160 mm Hg and/or diastolic blood pressure >95 mm Hg and/or the use of antihypertensive medication.
§Fasting glucose level >7 mmol/L or the use of antiglycemic medication or insulin.

RESULTS

Patient characteristics. A total of 607 white Dutch PAD patients were analyzed. Eleven percent (n = 67) of the patients had critical limb ischemia. Patient characteristics are given in Table I. The patients had an atherosclerotic risk factor profile, as seen in this table. It also became evident that the patients had extensive atherosclerotic disease, because 273 patients (45%) presented with manifestations of atherosclerosis in at least 1 additional vascular territory besides peripheral, thus illustrating the systemic nature of atherosclerosis.

***TLR4* and *CD14* genotyping.** Table II shows the genotype, carrier, and allele frequency of the *TLR4 +896 A>G* SNP in the PAD patients. Among our patients, there seemed to be a significant relationship between *TLR4 +896 G* allele carriership and the extent of clinically relevant atherosclerotic disease. The average extent-of-atherosclerosis score was higher in patients with a polymorph (G) allele compared with patients homozygous for the wild-type *TLR4* allele (1.8 vs 1.5; $P = .01$; Mann-Whitney *U* test). *TLR4 +896 G* carriers also had significantly more frequently (odds ratio, 2.2; $P = .02$; χ^2 test) extended clinically relevant atherosclerotic disease (more than two vascular territories affected). Trend analysis showed that the *TLR4 +896 G* allele frequency statistically significantly increased with the number of vascular territories affected by clinically relevant atherosclerosis (Table II). In a multivariate lo-

Table II. Genotype, allele, and carrier frequencies of the *TLR4 +896 A>G* alleles in atherosclerotic patients

Variable	*n*	*Genotype*			*Carrier frequency*	*Allele frequency*
		AA	AG	GG		
One affected vascular territory (PAD only)	334	307	27	0	8.1%*	4.0%
Two affected vascular territories	204	180	23	1	11.8%*	6.1%
Three affected vascular territories	67	54	13	0	19.4%*	9.7%
Four affected vascular territories	2	2	0	0	0.0%*	0.0%
Total	607	543	63	1	10.5%	5.4%

TLR4, Toll-like receptor 4; *PAD,* peripheral arterial disease.
Extended atherosclerotic disease (more than two vascular territories affected [n = 69] was significantly associated with *TLR4 +896 G* carriership (odds ratio, 2.22; $P = .020$; χ^2 test).
Carrier frequency is the percentage of patients with at least one polymorphic (G) allele (ie, patients with *AG* or *GG* genotypes).
Allele frequency is the ratio of the number of G alleles divided by the total number of alleles in the population.
*Trend analysis: *TLR4 +896 G* allele carrier frequency increases with the number of vascular territories affected (one to two to three or four): *P* trend, .01 ($\chi^2 = 7.2$).

Table III. Stepwise forward computed multivariate logistic regression model demonstrating the relationship between *TLR4 +896 G* carriership and the extent of clinically relevant atherosclerotic disease

Independent variable	*B*	*SE*	P *value*	*OR*	*95% CI*
Asp299Gly allele	0.735	0.371	.048	2.085	1.01-4.31
Hypertension	1.205	0.352	.001	3.338	1.68-6.65
Age	0.639	0.300	.033	1.895	1.10-3.41
Constant	−3.251	0.362	.001		

TLR, Toll-like receptor; *OR,* odds ratio; *CI,* confidence interval.
The dependent variable was extensive atherosclerotic disease (ie, more than two vascular territories affected; n = 607).
Variables excluded from the equation were sex, smoking, diabetes, hypercholesterolemia, and a positive family history.
B is the B-coefficient, an estimation of the change in the dependent variable that can be attributed to a change of one unit in the independent variable, and in fact is the natural log of the odds ratio.

Table IV. Allele and carrier frequencies of the polymorphic *CD14 −260 C>T* allele in atherosclerotic patients

Variable	*n*	*Genotype*			*Carrier frequency*	*Allele frequency*
		CC	CT	TT		
One affected vascular territory (PAD only)	334	88	169	77	73.7%	48.4%
Two affected vascular territories	204	52	112	40	74.5%	47.1%
Three affected vascular territories	67	16	35	16	76.1%	35.0%
Four affected vascular territories	2	0	2	0	100%	50.0%
Total	607	156	318	133	74.3%	48.1%

PAD, Peripheral arterial disease.
Extended atherosclerotic disease (more than two vascular territories affected) is not related to CD14 polymorphism.
Carrier frequency is the percentage of patients with at least one polymorphic (G) allele (ie, patients with the *AG* or *GG* genotype).
Allele frequency is the number of G alleles divided by the total number of alleles in the population.

gistic regression model, the association between the *TLR4 +896 A>G* SNP and extended atherosclerotic disease (more than two vascular territories affected) persisted after correction for relevant confounders (odds ratio, 2.1; $P < .05$; Table III).

The *CD14* polymorphism was very common (Table IV). Approximately 22% of patients had the TT genotype, and approximately 50% were heterozygous for the *CD14* polymorphism. In contrast to the *TLR4* polymorphism, the *CD14* SNP was not related to the extent of atherosclerotic disease in univariate analysis.

Carrier trait analysis. Finally, we performed a CD14/TLR4 carrier trait analysis in relation to the extent of atherosclerotic disease. Forty-four patients were carriers of the *TLR4 G* allele in combination with the *CD14 T* allele, and 14 patients had the *TLR4 G* allele in combination with the *CD14 TT* genotype. Carrier trait analyses for the *TLR4* and *CD14* SNPs studied showed a trend toward association with the extent of atherosclerotic disease, but this association failed to reach statistical significance in univariate logistic regression analyses (Table V). However, in the multivariate logistic regression model, when cardiovascular risk factors, carriership of the *TLR4 G* allele, the *CD14 TT* genotype, and the combination of the *TLR4 G* allele and the *CD14 TT* genotype were entered as independent variables, only the interaction variable *TLR4 G* allele/

Table V. Association between the carrier trait of *TLR4* and *CD14* SNP and the extent of atherosclerotic disease

	*TLR4-CD14, xG-xT**		*TLR4-CD14, xG-TT†*	
Extent of atherosclerotic disease	−	+	−	+
Two or fewer territories affected (n = 538)	503	35	528	10
More than two territories affected (n = 69)	60	9	65	4
All patients	563	44	593	14

TLR, Toll-like receptor; SNP, single nucleotide polymorphism.
*Univariate logistic regression analysis: $P = .054$; odds ratio, 2.156 (95% confidence interval, 0.99-4.70).
†Univariate logistic regression analysis: $P = .052$; odds ratio, 3.249 (95% confidence interval, 0.99-10.67).

Table VI. Stepwise forward computed multivariate logistic regression model demonstrating the relationship between the carrier trait of *TLR4 + 896 G* allele carriership (xG) and *CD14 − 260 TT* genotype *(TT)* and the extent of atherosclerotic disease

Independent variable	*B*	*SE*	P *value*	*OR*	*95% CI*
TLR4(xG)-CD14(TT)	1.434	0.665	.031	4.194	1.14-15.44
Hypertension	1.184	0.352	.001	3.269	1.64-6.52
Age	0.687	0.303	.023	1.988	1.10-3.60
Constant	−3.210	0.359	.001		

TLR, Toll-like receptor; *OR,* odds ratio; *CI,* confidence interval.
The dependent variable was extensive atherosclerotic disease (ie, more than two vascular territories affected; n = 607).
Variables excluded from the equation were sex, smoking, diabetes, hypercholesterolemia, positive family history, *TLR4 + 896 G* allele carriership, and *CD14 − 260 TT* genotype.
B is the B-coefficient, an estimation of the change in the dependent variable that can be attributed to a change of one unit in the independent variable, and in fact is the natural log of the odds ratio.

CD14 TT genotype (odds ratio, 4.2; $P = .03$) survived as an independent predictor of extensive clinically relevant atherosclerotic disease (ie, more than two vascular territories affected by atherosclerosis; Table VI).

DISCUSSION

In this study, we assessed the effect of the common *TLR4 + 896 A>G* and *CD14 − 260 C>T* polymorphisms on the extent of atherosclerosis in patients with PAD. With a multivariate logistic regression model, our data showed that the carrier trait profile *TLR4 G* allele in combination with the *CD14 TT* genotype had the strongest effect on the extent of atherosclerotic disease.

In this study, the overall *TLR4 + 896 G* allele frequency in white Dutch patients with symptomatic PAD was approximately 10%. This was comparable to our earlier reported *TLR4 + 896 G* allele frequencies in white Dutch women with or without tubal pathology,[23] to the *TLR4 + 896 G* allele frequencies in male patients with angiographically documented coronary atherosclerosis,[11] and to the frequencies in patients with meningococcal disease reported by Read et al.[24] Surprisingly, we did observe an interesting, significant partitioning in *TLR4 + 896 G* allele frequency: approximately 8% in patients with PAD only, 12% in patients with two vascular territories affected by clinically relevant atherosclerosis, and approximately 19% in patients with extensive clinically relevant atherosclerotic disease affecting three vascular territories. This represented a significant trend. This seems contradictory to earlier reports showing that the *TLR4* polymorphism protected against the development of early carotid plaque[8] and unstable coronary events.[10] In contrast to these earlier studies, we did not limit our analysis of the relationship between *TLR4* SNPs and atherosclerosis to coronary events or carotid plaques only. Instead, to take into account the systemic nature of clinically relevant atherosclerosis, we considered manifestations of atherosclerosis in the coronary, cerebral, and peripheral circulation and aorta. After all, approximately 50% of PAD patients have concomitant atherosclerotic disease affecting the coronary or cerebral circulation.[25,26] Alternatively, a significant proportion of patients with CAD have PAD.[27] To measure the total atherosclerosis load, techniques that detect symptomatic and asymptomatic atherosclerotic plaques, such as full-body electron beam computed tomographic scans[28] or electrocardiogram-gated, T2-weighted turbo spin echo magnetic resonance imaging of the thoracic and abdominal aorta,[29] may be used. Nevertheless, atherosclerotic lesions are a common occurrence in asymptomatic individuals, and not all atherosclerotic plaques will lead to clinical cardiovascular manifestations.[30] To determine whether specific genetic determinants were likely to be clinically relevant risk factors for atherosclerotic disease, we explored the relationship between SNPs and clinically relevant atherosclerosis. Therefore, the chosen outcome measure (the extent of clinically relevant atherosclerosis) took into account the systemic nature of atherosclerosis and incorporated only clinically relevant manifestations of atherosclerotic disease. Evidently, measures such as baseline electrocardiogram, stress tests, baseline carotid duplex scans, and cerebral

imaging may result in a higher incidence of (clinically silent) CAD and CVD. This might influence the association between the studied polymorphism and the extent of atherosclerotic disease. Bearing in mind the size of the analyzed cohort, it is expected that the used outcome measure may underestimate the extent of clinical relevant atherosclerosis in all patients regardless of their genetic makeup. Nevertheless, caution in the interpretation of the results should be undertaken, not in the least considering the low frequency of the carrier trait *TLR4 G* allele carriership/*CD14 TT* genotype (n = 14) in the studied population.

The early steps in atherogenesis often represent a response of the innate immune system to stimuli such as the accumulation and modification of lipoproteins in the arterial intima, whereas the progression of atherosclerosis depends on inappropriate activation of the innate and acquired immune system by both endogenous and exogenous stimuli.[2] The *TLR4 + 896 A>G* polymorphism may very well be associated with delayed development of early atherosclerotic plaques, theoretically through a blunted innate immune response, thereby explaining the inverse relationship observed between this polymorphism and carotid intima-media thickness and ultrasonographically detected, asymptomatic carotid plaque.[8] However, once the initial steps in atherogenesis have occurred, as is the case in PAD patients with an average age of 65 years, this *TLR4* polymorphism involved in the clearance of endogenous and exogenous (bacterial) atherogenic stimuli may be associated with exacerbated development of advanced atherosclerotic lesions, as shown in our study. The interaction between the *TLR4* polymorphism and atherosclerosis may also be influenced by the patient's pharmacotherapy. Boekholdt et al[11] showed that the *TLR4 + 896 G* allele indicated a significantly lower risk of cardiovascular events only for pravastatin users. Furthermore, the pravastatin-related risk reduction was more pronounced for *TLR4 + 896 G* allele carriers.

The CD14 promotor region polymorphism has been reported to enhance transcriptional activity of the *CD14* gene.[31] The TT genotype is associated with higher serum levels of sCD14, increased density of CD14 on monocytes, higher prevalence of *Chlamydia pneumoniae* infections, and enhanced chlamydia-stimulated tumor necrosis factor α production.[32,33] The *CD14 −260C>T* polymorphism has been associated with carotid artery intima-media thickness[12] and increased stroke risk[13] and acute myocardial infarction.[14,15] In contrast, Ito et al[16] and Longobardo et al[17] showed no relationship between this polymorphism and CVD or myocardial infarction, respectively. Likewise, using a nested case-control study within a large prospective cohort of apparently healthy individuals, Zee et al[18] demonstrated a lack of association between the *CD14 −260 C>T* polymorphism and (thromboembolic) stroke. Similarly, among the patients with PAD in our study, the *CD14* SNP was not related to the extent of atherosclerotic disease.

A considerable number of SNPs have been identified in innate immunity genes belonging to the TLR response pathway, among which are 44 SNPs in *TLR4* and 37 in *CD14*.[34] Therefore, when the role of specific SNPs is analyzed in relation to the severity of a specific disease, it is imperative to consider polymorphisms with functional implications that fit into a certain pathogenic paradigm. Otherwise, bearing in mind the considerable number of SNPs, there is a considerable possibility that statistically significant associations are described that may be based on chance only. Although it is accepted that the *CD14 −260 C>T* polymorphism is functional, questions remain regarding the functionality of the *TLR4 + 896 A>G* polymorphism. Even though the homozygous genotype is functional,[7] the heterozygous genotype, which has been associated with atherosclerotic disease in several studies, presents no deficit in the recognition of LPS.[35] However, this does not exclude the possibility that the heterozygous genotype is functional for other agonists that have not been tested on functionality in this heterozygous type, including human and chlamydial heat shock protein 60. Considering the complexity of the innate immune system and its high degree of genetic variation, a significant number of collateral pathways may exist for innate immune responses that differ in their cofactor requirements and their pattern-recognition specificities. Ideally, all pathways should be taken into consideration, and carrier traits instead of individual SNPs should be regarded when the genetic predisposition of the innate immune system is studied in relation to atherosclerosis and other inflammatory diseases, which are all multifactorial and polygenic diseases.

To illustrate this, it has recently been shown that carriage of multiple proinflammatory polymorphisms conferred a greater risk of noncardia gastric cancers, with odds ratios increasing from 2.8 for one to 27.3 for more than three high-risk SNPs.[19] In this study, we analyzed the combined effect of polymorphisms in two components of the innate immune system on the extent of atherosclerotic disease in patients with advanced atherosclerosis. Intriguingly, the combination of the *CD14* SNP resulting in transcriptional activity of the *CD14* gene[31] and carriership of the *TLR4 + 896 G* allele was related to the extent of clinically relevant atherosclerotic burden in patients with PAD. Failure to take into consideration SNPs in both genes may account for the contradictory results when *CD14* and *TLR4* polymorphisms were studied individually with regard to atherosclerosis. Similarly, failure to correct for relevant cardiovascular risk factors is another important aspect that has to be taken into account in this kind of study. In our population, the significance of the association between the combined *TLR4/CD14* carrier trait and the extent of atherosclerotic diseased was strengthened after correction for relevant risk factors (Table V).

In conclusion, considering the importance of (innate) immune responses in the development of atherosclerosis, our data provide a possible explanation for interindividual susceptibility to atherosclerosis based on genetic variability of a combination of genes involved in innate immune regulation. A carrier trait of a combination of *TLR4* and *CD14* SNPs, rather than each polymorphism individually,

was associated with the extent of atherosclerotic disease by using a multivariate logistic regression model.

AUTHOR CONTRIBUTIONS

Conception and design: TV, FRMS, SAM
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